Human mucosal IgA in health and disease

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Chapter 5

Early bacterial dependent induction of inducible nitric oxide synthase (iNOS) in epithelial cells upon transfer of $CD45RB^{high}$ $CD4^{+}$ T cells in a model for experimental colitis.

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Abstract

Both the role of inducible nitric oxide synthase (iNOS) in the development of inflammatory bowel disease (IBD) as well as the molecular details governing its mucosal induction remain unclear. In the present study we evaluated the role of the residing intestinal microflora in the induction of epithelial iNOS upon transfer of $CD45RB^{high}$ $CD4^{+}$ T cells to SCID mice. To this end, CB-17 SCID mice were reared with conventional flora (CNV) or germfree CB-17 SCID mice were mono-associated with *Helicobacter.muridarum*, act A (-) mutant *L. monocytogenes*, segmented filamentous bacteria (SFB), or O. anthropi. Within 2 weeks CNV SCID mice injected with $CD45RB^{high}$ $CD4^{+}$ T cells showed a focal, epithelial iNOS expression on the apical site of villi that preceded the infiltration of $CD4^{+}$ cells and cytokine production followed by extension of this expression to the entire surface along the whole crypt axis as the colitis progressed. SCID mice monoassociated with *H.muridarum* developed a severe colitis and showed high epithelial iNOS expression. CNV-SCID mice without T cells and SCID mice mono-associated with SFB did not show any iNOS expression, whereas SCID mice mono-associated with act A(-) mutant *Listeria. monocytogenes* and *Ochrobactrum. anthropi* showed some scattered epithelial iNOS staining on the apical site of a few villi, but none of these mice developed colitis. These findings demonstrate that the expression of epithelial iNOS is highly bacterium-specific and correlates with the severity of disease, suggesting an important role for this enzyme in the development of IBD.

5.1 Introduction

The inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are chronic inflammatory conditions of the human gastrointestinal tract. Studies in animal models suggest that IBD is due to an aberrant mucosal T cell response to gut bacteria. Several animal models have shown that both T helper cells and bacterial flora are needed to induce disease (Strober *et al*., 2002). The bacterial species and lymphoid interactions involved, however, remain subject to intensive discussion. In a mono-association study we showed that out of five non-pathogenic bacteria, only *H muridarum* was capable of provoking an accelerated colitis when compared to conventionally reared (CNV) mice after transfer of CD45RB^{high} CD4^{+} T cells to combined immunodeficient (SCID) mice (Jiang *et al*., 2002). A striking feature of *H muridarum* in comparison to many other bacterial species is that its ecological niche is in close contact with the epithelial cells of the crypts (Phillips and Lee, 1983). Therefore we hypothesized that epithelial cell activation by *H muridarum* might be an important trigger in the development of colitis in *H muridarum*-associated mice.

In patients with active IBD, there is a strong expression of inducible nitric oxide synthase (iNOS) at the apical side of epithelial cells (Dijkstra *et al*., 1998). The induction of iNOS is mediated by the nuclear factor κB (NF-κB) and it is known that some bacteria can directly activate NF-κB whereas other bacteria silence the epithelial NF-κB response to avoid epithelial cell activation (Naumann, 2000). It is at present neither known whether this induction is an important causative factor for the disease nor is it clear whether bacterial colonisation per se is associated with epithelial iNOS expression or whether the induction of iNOS is associated with specific bacterial types.

These considerations prompted us to compare the induction of iNOS after colonisation of the mouse intestine by different microflora. To this end we investigated whether epithelial iNOS expression and the development of colitis in CNV reared and mono-associated SCID mice after CD45RB^{high} CD4^{+} T cell transfer and generated a kinetic study relating epithelial iNOS expression to T cell infiltration and pro-inflammatory cytokine production in CNV and mono-associated SCID mice. We demonstrate an early focal expression of iNOS in epithelial cells preceding the T cell infiltration and pro-inflammatory cytokine production in CNV SCID mice after transfer of naïve T cells. At the terminal stages of colitis both CNV SCID mice as well as *H muridarum* mono-associated SCID mice showed strong iNOS expression along the whole crypt axis. In contrast, there was no or very low expression of iNOS in epithelial cells in SCID mice mono-associated with bacteria that did not induce colitis upon transfer of naïve T cells. These results demonstrate that bacteria specific epithelial cell activation and

subsequent iNOS induction and NO production is an early event in the development of colitis in the CD45RB^{high} CD4^{+} T cell transfer model of IBD.

5.2 Materials and Methods

5.2.1 Animal model

We used the CDRB45^{high} CD4^{+} T cell transfer model in SCID mice as described earlier (Jiang *et al*., 2002). Briefly, C.B-17 SCID mice were reared under conventional (CNV) or mono-associated conditions. Three weeks after stable colonization of germfree C.B17 SCID mice (>1010 bacteria/gm feces) with segmented filamentous bacterium, an avirulent actA (-) mutant DP-L1942 of *L monocytogenes* or after colonization by *O anthropi*, 5-10x105 CDRB45^{high} CD4^{+} T cells were injected intraperitoneally into the mono-associated SCID mice. For the kinetic study of epithelial iNOS expression in conventionally reared SCID mice 2 mice were sacrificed 1,2,3,4,5,7,8 and 13 weeks after T cell transfer. Mono-associated mice were sacrificed 6 weeks (*H. muridarum*) or 11 weeks (SFB, *L. monocytogenes and O. anthropi*) after T cell transfer. Normal BALB/c, conventionally and germfree reared SCID mice that did not receive T cells were used as controls. All experiment were approved by the animal welfare board.

5.2.2 Histology

Specimens of the large intestine were embedded in OCT compound (Miles Inc, Elkhart, IN) and frozen in 2-methyl-butane with dry ice. Longitudinal sections (4μM) were fixed with 4% paraformaldehyde, rinsed in PBS, and stained with hematoxylin-eosin.

5.2.3 Immunohistochemistry

For immunohistochemistry, 7 μm cryostat sections were cut, dried and fixed in acetone for 10 min at room temperature. For iNOS detection a rabbit polyclonal antibody developed in our laboratory was used (Vos *et al*., 1997). For CD4 detection a monoclonal biotin conjugated antibody clone H129.19 (Pharmingen,USA) was used. Slides were incubated with the polyclonal iNOS antibody (1:300) or monoclonal CD4 antibody (1: 50) in PBS containing 1% BSA for 60 min at room temperature. Subsequently, endogenous peroxidase activity was blocked by incubating for 30 min in PBS containing 0.075% H_2O_2. For iNOS

detection, peroxidase conjugated goat anti-rabbit Ig (1:50) and peroxidase conjugated rabbit anti-goat Ig (1:50) all from Dako (Glostrup, Denmark) were used as secondary and tertiary antibodies. For CD4 detection streptavidin-peroxidase (Southern Biotechnology Associates,USA) was used as secondary step. Color was developed using 3-amino-9-ethylcarbazole (10mg/2.5mL dimethylformamide in 50 ml 0.05 mol/L acetate buffer pH 4,9) containing 0,03% H_2O_2 for 10 minutes at room temperature. Counterstaining was performed with haematoxylin, and the slides were covered with Kaiser's glycerin-gelatin.

5.2.4 RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated from tissue specimens using Trizol reagent (Sigma-Aldrich Zwijndrecht, Netherlands) according to manufacturer's instructions. Reverse transcription was performed on 5 μg of total RNA using Oligo-dT primers (Invitrogen, Breda, Netherlands) in a final volume of 30 μl. Polymerase chain reaction (PCR) on cDNA was performed with Taq polymerase (Invitrogen, Breda, Netherlands) on the Biometra PCR system. The PCR primers for mice iNOS, TNF-α, IL-1β, IFN-γ, Gro, MMIP and β-actin were selected from multiple exons and are depicted in table 1. The cycling program was 94°C for 2 min, 58°C for 60 seconds, 72°C for 60 seconds for the first cycle and 94°C for 30 sec, 58°C for 60 seconds, 72°C for 60 seconds for 30 cycles. The level of mRNA expression is expressed relative to the β-actin level.

	Sense	Anti-sense	Bp
iNOS	5'-ggtatgctgtgtttggccttt-3'	5'-ggctggacttttcactctgc-3'	390
TNF-α	5'-ctcttcaagggacaaggctg-3'	5'-cggactccgcaaagtctaag-3'	253
IL-1β	5'-caggcaggcagtatcactca-3'	5'-aggccacaggtattttgtcg-3'	350
IFN-γ	5'-actggcaaaaggatggtgac-3'	5'-tgagctcattgaatgcttgg-3'	237
Gro	5'-gctgggattcacctcaagaa-3'	5'-tggggacacctttagcatc-3'	169
MMIP	5'-agtgaactgcgctgtcaatg-3'	5'-ttcagggtcaaggcaaactt-3'	153
β-actin	5'-cctaaggccaaccgtgaaaag-3'	5'-tcttcatggtgctaggagcca-3'	672

5.2.5 Statistical Analysis

Significant differences between mean values were determined by Student t test. $P<0.05$ was considered significant.

5.3 Results

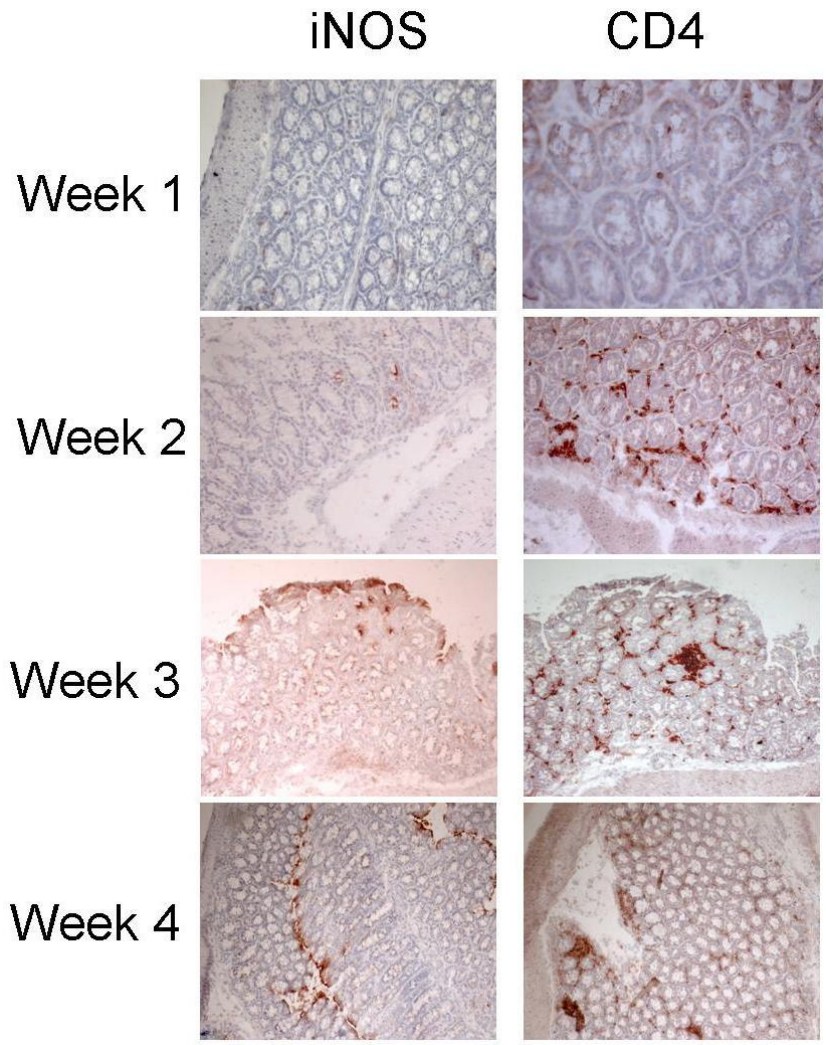
5.3.1 Kinetics of T cell infiltration, cytokine production and epithelial iNOS induction in CNV-reared SCID mice after CD45RB^{high} CD4^{+} T cell transfer.

T cell transfer in SCID mice is generally considered a rodent model for IBD, mimicking important aspects of the disease. CNV BALB/c showed sporadic CD4^{+} T cells, whereas none were observed in the lamina propria of CNV SCID. Upon T cell transfer, CNV SCID mice show small aggregates of CD4^{+} T cells near the muscularis propria (Figure 1 right panel). After 4 weeks the cells are no longer present near the muscularis but now small aggregates are present in the lamina propria, sometimes close to epithelial cell compartment. At 8 weeks, substantially more and larger lamina propria conglomerates of CD4^{+} T cells are observed. The end-stage of colitis is characterised by a lamina propria completely filled with conglomerates of CD4^{+} T cells. Thus the histology observed in this model is consistent with an IBD like inflammatory reaction in the colon.

This notion is supported by RT-PCR experiments for the pro-inflammatory cytokines IL-1β, TNF-α, IFN-γ and chemotactic cytokines Gro and mMIP. In these experiments we observe equal levels of TNF-α and IFN-γ in BALB/c and SCID mice that did not receive CD4^{+} T cells (Figure 2). IL-1β and mMIP levels are reduced in untreated SCID mice compared to BALB/c mice. The mRNA of the pro-inflammatory cytokines IL-1β, TNF-α and INF-γ begins to rise 3 weeks after T cell transfer and shows a biphasic response with high peaks around 6 and 10 weeks Figure 2). The chemotactic cytokine Gro shows the same pattern, whereas another chemokine, mMIP shows only an increase in the terminal phase of colitis (Figure 2). Subsequently experiments were initiated to investigate the expression of iNOS during such inflammation.

Despite a critical function of iNOS in mucosal immune responses, the relationship between its expression and the composition of the gut flora is poorly understood. CNV BALB/c and CNV SCID mice do not show any iNOS expression in their colon tissue (Figure 3, left panel). CNV SCID mice show focal epithelial iNOS already 2 weeks after T cell transfer (Figure 1, left panel). The iNOS expression is on the apical site of the enterocytes on the top of the villi and not in the crypt epithelial cells. In week 3 and 4 after T cell transfer larger areas of epithelial cells show iNOS expression but it is still confined to the top of the villi. From week 5

also crypt epithelial cells show iNOS expression. At week 8 the intensity of the iNOS expression is further increased and involves almost the whole epithelial surface (figure 3, right panel).



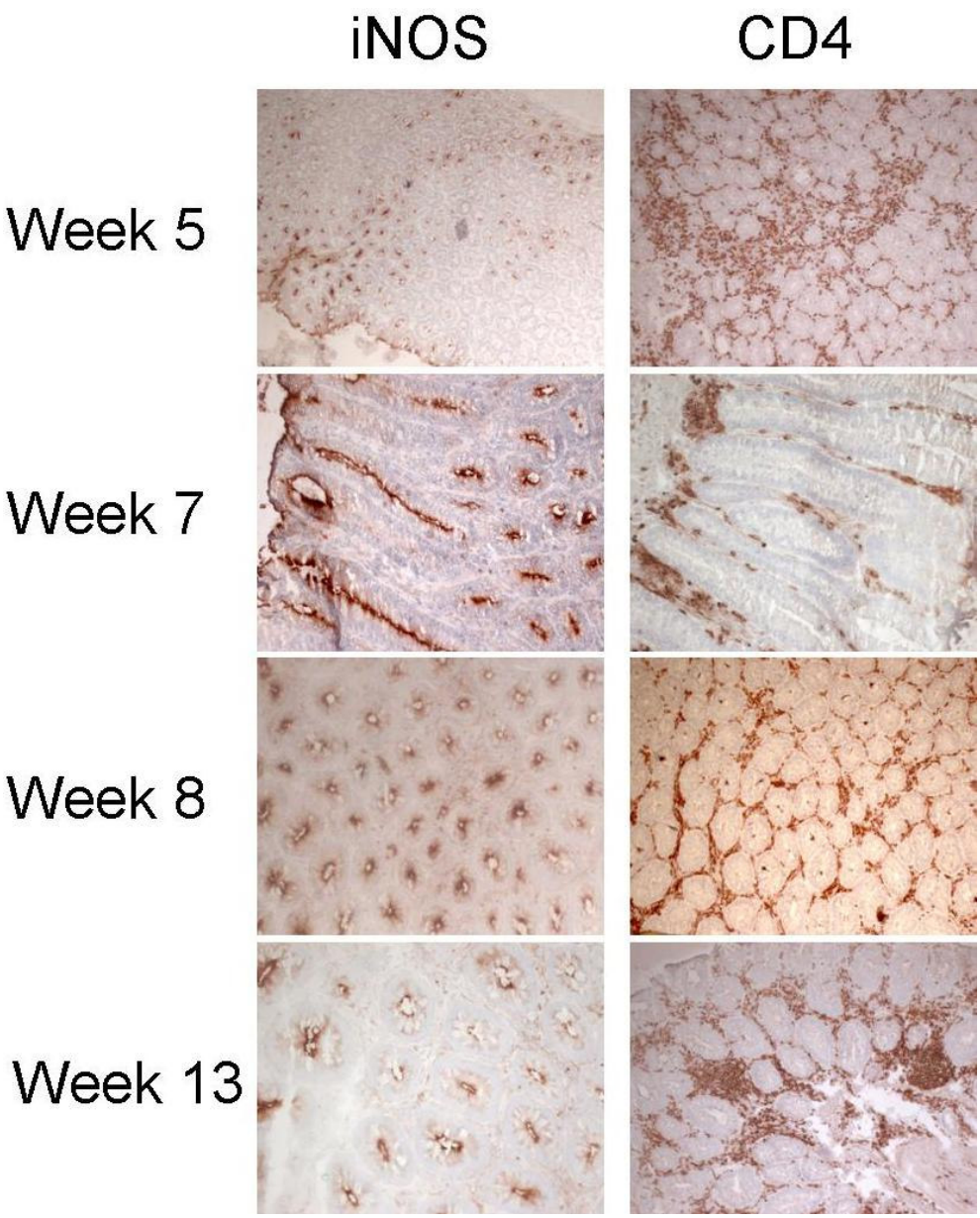


Fig. 1 Immunohistochemistry of the colon for iNOS (left panel) and CD4 (right panel) 1, 2, 3, 4, 5, 7, 8, 13 weeks after transfer of CD45RB^{high}CD4^{+}T cells into Severe Combined Immunodeficiency mice (SCID) reared with conventional flora (CNV). The epithelial cells express iNOS 2 weeks after T cell transfer. In the first

weeks this expression is focal and confined to top of the crypts, in the weeks thereafter the iNOS expression is diffuse and also present in epithelial cells in the crypts. CD4$^+$ T cells are first present near the muscularis propria (right panel) and expand into the lamina propria after 4 weeks. After 8 weeks the lamina propria is filled with conglomerates of CD4$^+$ T cells.

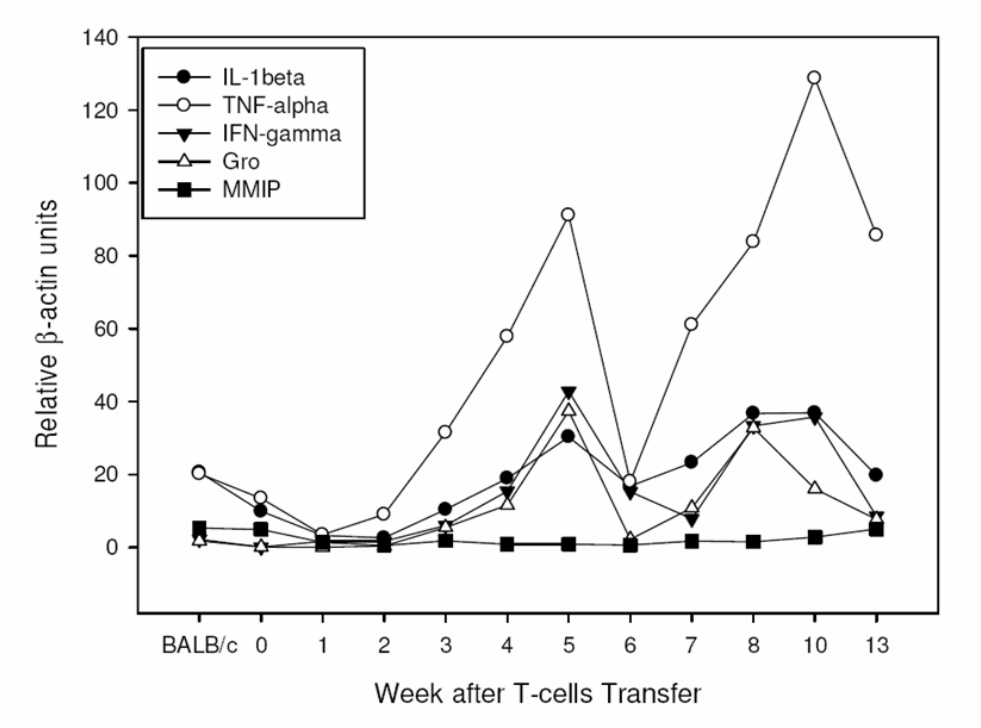


Fig. 2 RT-PCR for mRNA of IL-1β, TNF-α, IFN-γ and the chemokines Gro and mMIP. Equal levels of TNF-α and IFN-γ mRNA are observed in BALB/c and SCID mice that did not yet receive CD4$^+$ T cells. IL-1β and mMIP were reduced in SCID mice compared to BALB/c mice. The mRNA levels of the pro-inflammatory cytokines IL-1β, TNF-α and INF-γ start to increase 3 weeks after T cell transfer and showed a biphasic response with high peaks around 6 and 10 weeks (figure 3). The chemokine Gro showed the same pattern, whereas another chemokine mMIP increased only in the terminal phase of colitis

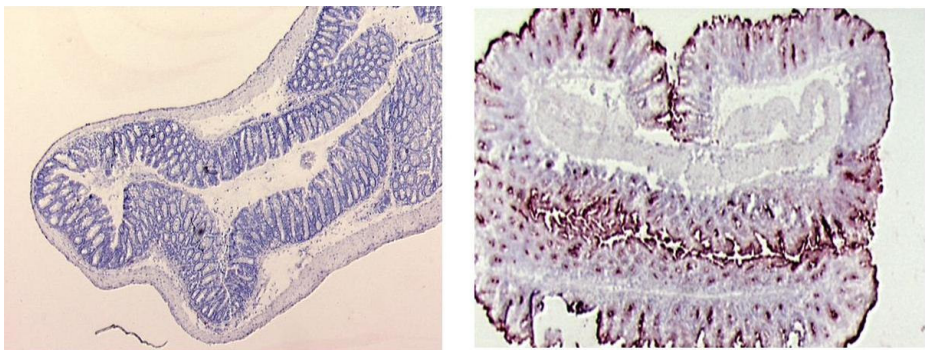


Fig. 3 Immunohistochemistry of the colon for iNOS without (left panel) and 8 weeks after (right panel) transfer of CD45RB^{high}CD4^{+}T cells into Severe Combined Immunodeficiency mice (SCID) reared with conventional flora (CNV).

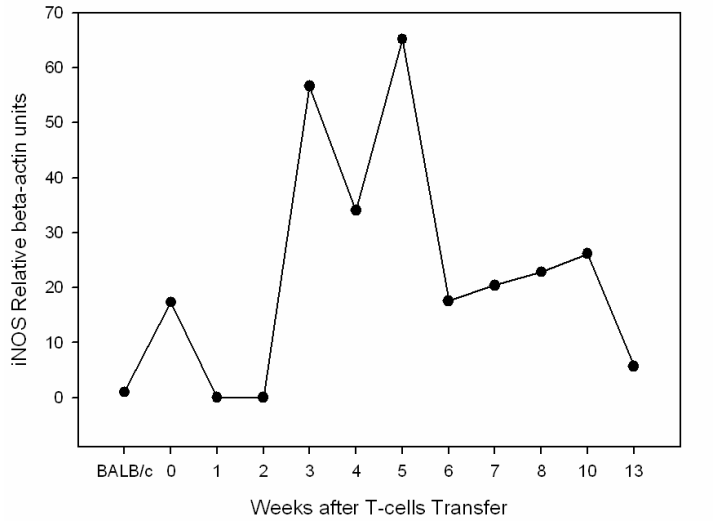


Fig. 1 RT-PCR for iNOS (mRNA expression relative to expression of β-actin) of the colon from CNV reared SCID mice colon 1, 2, 3, 4, 5, 7, 8, 13 weeks after transfer of CD45RB^{high}CD4^{+}T cells. The iNOS mRNA is detectable as early as 3 weeks after T cell transfer.

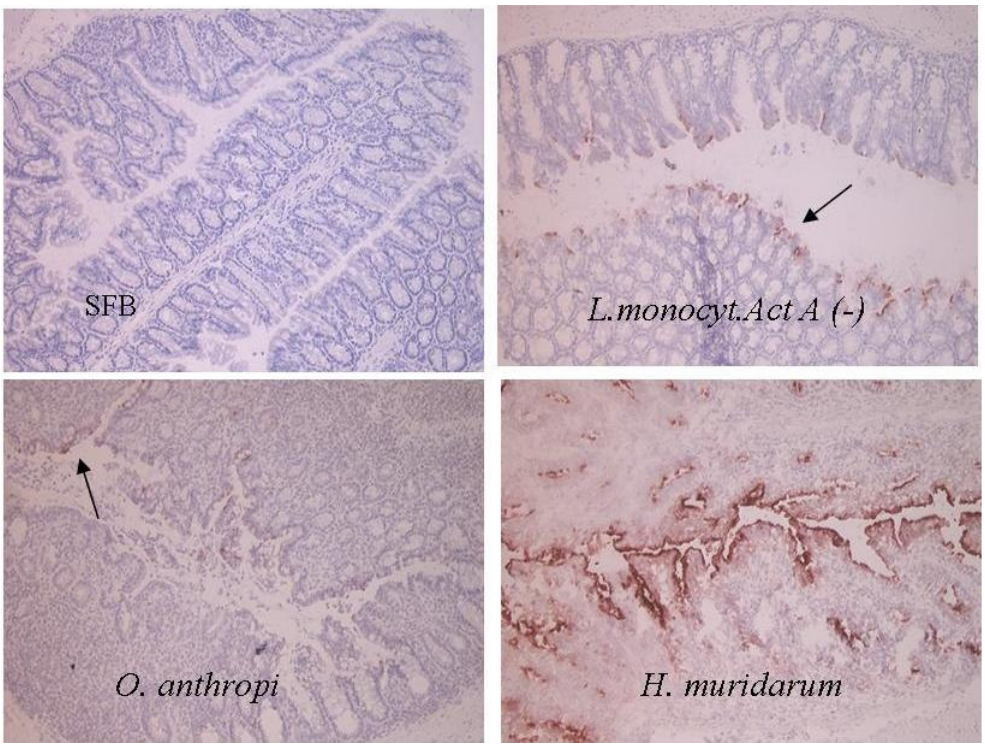


Fig. 2 Immunohistochemistry of the colon for iNOS 11 weeks and 6 weeks (for H.muridarum) after transfer of CD45RB^{high}CD4^{+}T cells into Severe Combined Immunodeficiency mice (SCID) monoassociated with SFB, L. monocytogenes Act A (-), O. anthropi or H. muridarum. SFB-monoassociated mice did not induce iNOS expression 11 weeks after T cell transfer. Mice mono-associated with Act A(-) L. monocytogenes and O. anthropi demonstrated a low level of very focal iNOS staining that was confined to the apical site of the enterocyte and was only present in enterocytes on the top of a few villi (arrows). However, mice mono-associated with H. muridarum showed at 6 weeks an intense and diffuse iNOS staining of enterocytes along the whole crypt axis.

At the end-stage of colitis the whole epithelial surface shows a diffuse but intense iNOS expression along the whole crypt axis. Inflammatory cells show no iNOS expression. RT-PCR analysis (Figure 4) confirms the immunohistochemistry results although the RT-PCR for iNOS was positive shortly after the focal epithelial staining was already seen at 2 weeks. The inflammatory reaction in colon following T cell transfer correlates with the induction of epithelial iNOS expression. Thus expression iNOS correlates with the severity of the mucosal inflammatory reaction.

5.3.2 Epithelial iNOS induction in mono-associated SCID mice after CD45RB^{high} CD4^{+} T cell transfer

Subsequently, we investigated different flora components for their effect on iNOS expression in IBD. Mice mono-associated with SFB do not show any iNOS expression 11 weeks after T cell transfer (figure 5). Mice mono-associated with Act A(-) L. monocytogenes and O. anthropi show very focal iNOS staining 11 weeks after T cell transfer. The iNOS staining is confined to the apical site of the enterocyte and is only present in enterocytes on the top of a few villi (figure 5). However, 6 weeks after T cell transfer, concomitant with clear clinical syptoms of IBD in mice mono-associated with *H. muridarum*, an intense and diffuse iNOS staining of enterocytes along the whole crypt axis is observed (figure 5). Hence specific flora components are markedly different with respect to inducing iNOS expression and the extent of the iNos expression correlates with the severity of the mucosal inflammation.

5.4 Discussion

In this study we show that epithelial cells of CNV reared SCID mice express iNOS early in the development of colitis after CD45RB^{high} CD4^{+} T cell transfer. The absence of epithelial iNOS expression in CNV reared SCID mice that did not receive naïve T cells demonstrates that both the bacteria and T cells are needed for epithelial iNOS expression and the development of colitis. The mono-association studies show that certain bacteria (e.g. SFB) do not induce iNOS and colitis, whereas other bacteria (e.g. *L. monocytogenes* act A(-), *O. anthropi*) induce a very low level of iNOS expression without causing full-blown colitis, whereas yet other bacteria (e.g. *H. muridarum*) give rise to strong epithelial iNOS induction and accelerated colitis as compared to CNV reared SCID mice. Thus iNOS expression in general correlates with the severity of mucosal inflammation.

The induction of iNOS is mediated by the nuclear transcription factor κB (NF-κB) in epithelial cells (Xie *et al*., 1994) and in other cells involved in intestinal colitis (Comalada *et al*., 2005). Our current data support this notion, iNOS expression correlates well with the severity of the colitis and the production of NF-κB-dependent cytokines. The observation that a combination of cytokines (IL-1β, IFN-γ, TNF-α) and endotoxin (LPS) is needed for the in vitro induction of iNOS in native colon cells and intestinal tumor cell lines may be interpreted as that epithelial cells of the colon are relatively resistant to cytokine induced NF-κB

activation (Jobin *et al*., 1997). More specifically a decreased IκB kinase (IKK) activity and a consequent resistance to IκBα degradation has been postulated as a protective response of intestinal epithelial cells, enabling the cells to remain quiescent in the hostile strongly pro-inflammatory colon environment (Jobin *et al*., 1997). However, this relatively unresponsive state still allows several particulary pathogenic enteroinvasive organisms such as Salmonella, Shigella, Listeria and Helicobacter species to directly activate NF-κB (Naumann, 2000) and induce iNOS and pro-inflammatory cytokines in intestinal epithelial cells (Witthoft *et al*., 1998). This may be an important defence response as the bacterial pathogenic genus Yersinia has developed delivery of virulence Yop factors capable of blocking the NF-κB mediated production of pro-inflammatory cytokines, thus preventing an anti-bacterial epithelial cell response (Schesser *et al*., 1998). It will be of interest to investigate whether reduced iNOS expression as a consequence of impaired NF-κB activation in Yersinia-infected cells is important in this reduced anti-bacterial epithelial cell response.

Apart from pathogenic bacteria there is also evidence that non-pathogenic bacteria in the normal gut can prevent epithelial NF-κB induction and hence contribute to the reduction of the mucosal immune response against normal gut bacteria. Therefore, the capability of bacteria to interfere with the epithelial NF-κB response might reflect its potential to induce or suppress IBD. Studies aimed at inhibition of NF-κB activation in IBD are promising (Dijkstra *et al*., 2002;Neurath *et al*., 1996) and the correlation observed in the present study between iNOS expression and the severity of the colitis calls for studies in which the importance of iNOS expression for mucosal inflammatory reaction in IBD is assessed directly. However, as shown by a conditional epithelial NF-κB knockout animal model an adequate epithelial NF-κB response is also an important anti-apoptotic response necessary for epithelial healing and repair, thus strategies directly aimed at inhibiting iNOS downstream of its induction by NF-κB may be more promising as future therapeutic avenues for dealing with IBD (Chen *et al*., 2003).

In this context it is important to keep in mind that although we observe a clear correlation between the epithelial iNOS response and the development of colitis, the exact role of iNOS and epithelial derived NO in the development of IBD is not known (Grisham *et al*., 2002). In relation to this, an increase in iNOS expression in colonic samples has been observed in some animal models (Camuesco *et al*., 2004). However, studies using inhibitors of NOS in experimental colitis are conflicting and show little improvement (Hogaboam *et al*., 1995;Rachmilewitz *et al*., 1995); no effects (Conner *et al*., 1995;Ribbons *et al*., 1997), or even worse effects (Pfeiffer and Qiu, 1995) on colitis probably due to the lack of iNOS specificity (i.e. also inhibition of endothelial NOS) of the inhibitors used. In addition, studies of experimental colitis in iNOS knockout mice also showed conflicting results even when the same experimental model was used (Beck *et al*.,

2004;Hokari *et al*., 2001;Krieglstein *et al*., 2001;McCafferty *et al*., 1997;McCafferty *et al*., 1999;vallance *et al*., 2004;Zingarelli *et al*., 1999). IL-10 knockout mice develop colitis spontaneously. Colitis developed at the same rate and intensity in IL-10 knockout mice and IL-10/iNOS double knockout mice (McCafferty *et al*., 2000). Considering the absence of macroscopic ulcerations in the presence of large amounts of NO in patients suffering from microscopic colitis a role of NO in diarrhoea and ulcer healing has been suggested (Lundberg *et al*., 1997). Indeed topical administration of the NOS inhibitor Ng-monomethyl-L-arginine (L-NMMA) reduced fluid secretion in patients with collagenous colitis (Perner *et al*., 2001) and a NO donating mesalazine derivative had an additional beneficial effect on TNBS induced colitis (Wallace *et al*., 1999). The reduced gastro-intestinal toxicity of NO-donating non steroidal anti-inflammatory drugs (NSAID) (Elliott *et al*., 1995) and aspirin (Fiorucci *et al*., 2003) are in agreement with a protective effect of NO on intestinal epithelial cells. Apart from the above-mentioned beneficial effects of NO in mucosal injury, NO can also inhibit NF-κB activation (Matthews *et al*., 1996). Therefore, high amounts of NO may participate in a negative feedback loop to block prolonged activation of NF-κB, thereby limiting chronic inflammation, in which case strategies aimed at inhibiting NO production may be self-defeating. In this context it must be kept in mind that NO itself is not be toxic to many bacteria as tcertain enteric bacteria contain nitrate reductase and produce NO by their own (Brittain *et al*., 1992). Importantly, however, as observed in septic patients, epithelial iNOS induction and NO production may cause increased intestinal permeability (Johnston *et al*., 1996). Indeed selective inhibition of iNOS in endotoxemic rats ameliorated mucosal permeability for dextran (MW 4000) (Unno *et al*., 1997) and reduced bacterial translocation (Sorrells *et al*., 1996). The absence of bacterial translocation in endotoxemic iNOS knockout mice further supports a pathogenic role of epithelial derived NO in sepsis. As long as there are no truly selective iNOS inhibitors available it will be hard to examine the exact role of epithelial iNOS induction and NO production in IBD. In this study however, we provide evidence that epithelial iNOS expression is an early and bacteria dependent event in the development of colitis in the $CD45RB^{high}$ $CD4^{+}$ T cell transfer model of IBD and that bacteria that cause colitis also induce high epithelial iNOS expression.

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